Journal of Ecobiotechnology 2/4 27-32, 2010
ISSN 2077-0464

Journal of Ecobiotechnology
http://journal-ecobiotechnology.com/

Biodegradation of BTEX (Benzene, Toluene, Ethyl Benzene and Xylene) Compounds by Bacterial Strain under Aerobic Conditions

Rajni Singh[1]* and S. Mary Celin[2]
[1]Amity Institute of Microbial Biotechnology, Amity University Campus, Sector, 125, Noida - 201301, UP, India
[2]Centre for fire, Explosives and Environment Safety, Brig. S.K. Majumdar Road, Timarpur, Delhi-110054, India

**Corresponding author, Email: rsingh3@amity.edu, Tel: 0120-4392900, Fax: 0120-4392295*

Keywords

BTEX
Microbial degradation
Aromatic hydrocarbons
GC analysis

Abstract

Petroleum aromatic hydrocarbons like benzene, toluene, ethyl benzene and xylene, together known as BTEX, share almost the same chemical structure. These aromatic hydrocarbons are widely used in different industries and are released in the environment in abundance thus, polluting the entire ecosystem. Recent bioremedial applications of microorganisms to treat these pollutants have shown significant results. This present work highlight a solvent tolerant bacterial strain Bb5 that degrades BTEX compounds as they all share a common chemical structure. The bacterial strain can tolerate and grow in presence of high concentration of BTEX (10% of toluene, 10% of benzene 2-5% ethyl benzene and 0.5% xylene). Gas chromatography results reveal that the strain is capable of degrading BTEX in different concentrations ranging 100 % in benzene and 80% in toluene within 48 and 72 hours. This property can be used for effluent treatment, solvent waste management.

1. Introduction

The petroleum hydrocarbon compounds are most common groundwater and soil contaminants. For the degradation of these compounds the bacteria should come in physical contact with the pollutants. Affinity of a bacterial strain towards a particular hydrocarbon ensures a wide choice of degradation. As most of the aromatic hydrocarbon pollutants have a similar structure it is possible that the bacterial strain showing affinity towards one hydrocarbon shows affinity towards other related aromatic hydrocarbon. Once cells are brought into close contact with pollutants, mechanisms like biofilm formation and surfactant production can come into play to increase the bioavailability and biodegradation of the hydrocarbon.

Because of the high solubility in water relative to petroleum hydrocarbons, BTEX (benzene, toluene, ethyl benzene and o-, m- and p-xylene) are found in the water-soluble fraction, and groundwater can transport them from tens to hundreds of meters down gradient of the contamination source [1, 2]. BTEX compounds are toxic to humans and are confirmed or suspected carcinogens; thus, the United States EPA classifies them as environmental priority pollutants [3], making their removal from polluted environments critical. The BTEX compounds are especially difficult to degrade because they lack an activating oxygen or nitrogen substituent group which would make oxidation of the ring more energetically feasible.

Biodegradation of BTEX compounds by microorganisms has shown promising results to overcome this problem. Bacteria that degrade BTEX compounds under aerobic conditions are widely distributed in nature. Studies on the metabolism and genetics of BTEX degraders suggest that mostly *Pseudomonas* species [4, 5, 6] or closely related *Ralstonia* and *Burkholderia* species [7], are involved in the degradation of BTEX compounds. In addition *Rhodococcus* [8, 9], *Marinobacter* [1] *Acinetobacter* [3], *Alcaligenes, Brevibacterium, Cladophialophora* sp. strain T1 [10] *Nocardia, Bacillus, Bordetella, Arthrobacter, Bradyrhizobium, Acidovorax, Agrobacterium, Aquaspirillum, Variovorax* and *Stenotrophomonas* were also detected as BTEX degraders in soil [11, 12]. A halotolerant *Streptomyces* sp., isolated from an oil field in Russia degraded crude petroleum

Several factors, such as pollutant concentration, active biomass concentration, temperature, pH, availability of inorganic nutrients and electron acceptors, and microbial adaptation, influence the rate and extent of biodegradation of BTEX. Since the BTEX compounds are known to occur as a mixture in contaminated sites, an organism that simultaneously degrades all the different components of BTEX is more desirable than an organism that degrades only some of the BTEX compounds.

The present study will highlight a solvent tolerant bacterial strain Bb5 that can degrade high concentration of BTEX compounds in a short time span. The morphological changes these compounds bring in the internal organelles of the bacterial strain were also studied.

2. Materials and Methods

Isolation of microorganisms

Soil samples were collected from different hydrocarbon rich areas in New Delhi and Noida, U.P., India. The soil samples were suitably diluted using standard serial dilution procedure and inoculated into 20 ml nutrient broth over laid with 1% v/v toluene in 100 ml sealed serum bottles and incubated at 37°C with constant shaking at 120 rpm orbital shaker for 5 days. 0.1% of these 5 days old inoculum were then plated in nutrient agar plates and incubated overnight at 37°C. Growing colonies were purified by repeated streaking on agar plates. The isolated strains were maintained on nutrient agar slants at 4°C.

Media and culture conditions

Inoculums of the isolated strains were prepared in nutrient broth (pH 7.5) by overnight incubation at 37°C and 120 rpm. 0.1% of each overnight grown culture was used to inoculate 20 ml culture media (over laid with 1% v/v to 10% v/v toluene) in 100 ml sealed serum bottles and incubated at 37oC with constant shaking at 120 rpm orbital shaker. The cultures in absence of the solvent and uninoculated media enriched with the solvent were used as control under similar condition.

Determination of BTEX tolerance

To determine the level of BTEX tolerance 1% of the overnight grown enriched cultures were inoculated in 20 ml nutrient broth supplemented separately with benzene (0.1-10%), toluene (0.1-10%), ethylene benzene (0.1-5%) and xylene (0.1-0.5%) in 100ml screw cap flask and incubated at 37°C under shaking (250 rpm) for 48 h. All the experiments were carried in duplicate.

Growth of bacteria in presence of BTEX

1% of the overnight grown enriched cultures, that showed tolerance towards high concentration of BTEX, were inoculated in 20 ml nutrient broth supplemented separately with benzene (0.1-10%), toluene (0.1-10%), ethylene benzene (0.1-5%) and xylene (0.1-0.5%) in 100ml screw cap flask and incubated at 37°C under shaking (250 rpm) for 48 h. Growth of the bacterial strains in presence of BTEX was determined by increase in O.D. at 660nm at a constant time interval of 8 h till 48 h. All the experiments were carried in duplicate.

Determination of BTEX degradation

The best BTEX tolerant strain was further analyzed to determine its rate of BTEX degradation by GC methods. 2% of the enriched bacterial culture was inoculated in four different 100ml capacity serum bottles filled with 20 ml of nutrient broth overlaid with 10% v/v of toluene & benzene, 0.5% xylene and 2% ethyl benzene separately. The bottles were then closed with Teflon-coated septa and aluminum caps and were incubated for 48h at 37°C under 250 rpm and degradation of BTEX was monitored by gas chromatography (Clarus500_APL/INS/73) analysis [1]. The bacterial strain in absence of BTEX and uninoculated media enriched with BTEX were used as control under similar condition. These experiments were carried out in duplicate.

3. Results

Isolation of microorganisms

About 24 hydrocarbon tolerant bacterial cultures were isolated from the above mentioned sites in presence of 1% (v/v) toluene. Most of the isolated strains were gram positive rods (Table 1). The isolated strains were further characterized on the basis of the BTEX tolerance level.

Table1: Morphological Characteristics and BTEX tolerance level by different bacterial strains

Bacterial strains	Gram Character	Morphology	Benzene (%)	Toluene (%)	Ethyl Benzene (%)	Xylene (%)
Aa1	(-)	Big rods, single / clusters	5	7	0.5	0.1
Aa2	(+)	Cocci in chains	7	5	0.5	0.1
Aa3	(+)	Rods	5	1	1	_
Aa4	(-)	Rods in clusters	2	1	0.2	0.2
Aa5	(+)	Rods in chains	5	5	2	_
Aa6	(+)	Rods	2	2	0.2	_
Ab2	(+)	Rods in big chains	7	5	5	0.1
Ab3	(+)	Rods in clusters	7	5	2	0.2
Ab4	(-)	Rods	5	7	2	0.2
Ab5	(+)	Cocci	5	7	2	0.1
Ab6	(-)	Rods	2	5	1	_

Ab7	(+)	Rods	5	2	1	_
Ab8	(+)	Cocci	5	2	1	0.1
Ab9	(+)	Rods	7	7	2	0.2
Ab10	(+)	Rods	5	7	2	0.1
Ac1	(-)	Rods	2	5	1	0.1
Bb5	(-)	Rods	10	10	5	0.5
Bb6	(+)	Cocci	7	10	2	0.2
Bb7	(+)	Rods	10	5	2	_
Bc1	(-)	Rods	7	7	1	_
C4	(-)	Rods in clusters	5	2	0.2	_
C5	(+)	Rods	2	5	0.5	0.1
C6	(+)	Cocci	7	5	1	0.1
C7	(+)	Rods in chains / clusters	10	10	5	0.5

Determination of BTEX tolerance

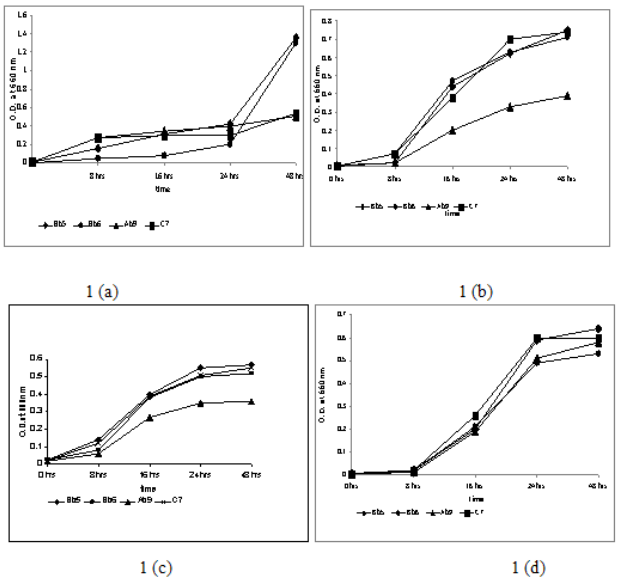
The 24 isolated strains showed different level of tolerance in presence of different concentration of BTEX compounds after 48h incubation. The best four strains (Bb5, Bb6, Ab9, C7) showing high tolerance level were screened. Bb5 and C7 showed tolerance in presence of 10% (v/v) toluene and benzene, 5% (v/v) ethyl benzene and 0.5% (v/v) xylene (Table 1).

Growth of bacteria in presence of BTEX

All the 4 strains were grown in presence of 1% to 10% (v/v) toluene (Fig1a). During growth, among all four strains, Ab9 and C7 showed the highest absorbance in initial (8h) hours of incubation but gradually in the later phase of incubation there is no constant increase in the absorbance. In contrast to this, all 4 bacterial strains exhibited almost the same growth pattern in presence of 1% to 10% (v/v) benzene (Fig1b) and 0.1% to 5% (v/v) ethyl benzene (Fig1c) where they exhibited maximum growth and absorbance in 8 to 24h of incubation. Growth of all the bacterial strains was also arrested in presence of more than 0.5% xylene (Fig1d).

The growth of all the bacterial strains showed high absorbance in 8h of incubation. These indicate that Ab9 and C7 had a very short log phase and a prolonged stationary phase in their growth cycle. While Bb5 and Bb6 had prolonged lag phase but gradually attained their log phase from 24 h of incubation with a sharp increase in their absorbance that continued till 48 h of incubation. However in presence of benzene (1 to 10% (v/v)) and ethyl benzene (0.1 to 5% (v/v)) all 4 bacterial strains were in their lag phase in the initial 8 hours. All of them showed the same growth pattern. None of the strains showed the absorbance of ≥ 0.95 in presence of 0.5% (v/v) xylene while all the bacterial strains exhibited the absorbance of 1.7 in absence of xylene after 48 h of incubation. They attained their log phase between 8 to 24 h of incubation and gradually entered the stationary phase from 24 h (Fig 1d).

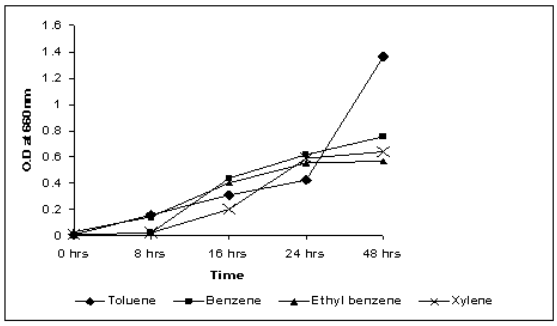
Figure 1: Growth curve of the four bacterial strains in presence of different BTEX components. (a) Growth of the four strains in presence of toluene, (b) Growth of the four strains in presence of benzene, (c) Growth of the four strains in presence of ethyl benzene, (d) Growth of the four strains in presence of xylene



1 (a) 1 (b)

1 (c) 1 (d)

Figure 2: Growth of Bb5 in presence of BTEX compounds

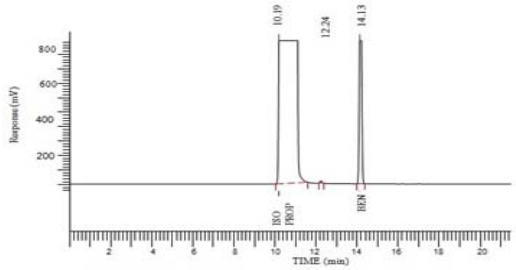


The bacterial cultures showed different growth patterns in presence of BTEX. Though all the four strains were able to grow in presence of high concentration (10%) of toluene and benzene, their growth has been restricted in presence of 5 % (v/v) ethyl benzene and very low concentration of xylene. Strain Bb5 showed the highest absorbance among all the 4 strains in presence of different BTEX compounds, hence it was selected to further determine the degradation of BTEX compounds (Fig 2).

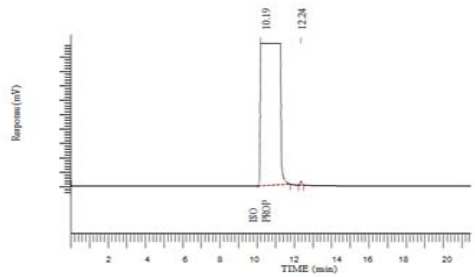
Determination of BTEX degradation

GC analysis revealed that Bb5 degrades different BTEX compounds at different rate in the same time span. The selected strain Bb5 was able to degrade 100% of benzene [5% (v/v)] (Fig 3a & b) and ethyl benzene [2% (v/v)] as the benzene and ethyl benzene peaks were completely absent in the GC chromatogram while toluene [5% (v/v)] and xylene [0.5% (v/v)] were degraded up to 80% and 70% respectively.

Figure 3: (a) GC analysis of control media having 5% (v/v) benzene, (b) GC analysis of media having 5% (v/v) benzene inoculated with Bb5



3(a)



3 (b)

4. Discussion

Very few isolated microorganisms are known to degrade BTEX compounds, probably because isolation techniques have focused largely on laboratory enrichment procedures using BTEX as the sole carbon and energy source. Kim et al. [13] have isolated about 80 bacterial strains from a gasoline-contaminated soil sample containing ≈4,500 mg/kg total petroleum hydrocarbons on MSB agar, using four different procedures. A total of 21 different bacterial phylotypes were identified by Berlendis et al [14] each community containing three to nine bacterial phylotypes.

The growth of the strains was acclimatized from 2 to 5% (v/v) ethyl benzene and 5% to 10% (v/v) benzene, as they exhibited good growth in presence of 2% benzene and ethyl benzene. Similar acclimatization process has also been reported for activated sludge in the microbial treatment of restaurant fats, oils and grease [15].

The different growth patterns of the bacterial cultures in presence of BTEX suggest that all the four cultures have utilized different components in different manner. The increase in the growth of bacterial strains and the difference on the lag phase seen when exposed over a long term to BTEX atmosphere indicates that BTEX-degrading organisms had become acclimatized to the concentrations that are lethal and extreme environment to normal bacteria. In the seawater, degrading bacteria of a relatively wide diversity were detected, including species of Pseudomonas, Rhodococcus, Exiguobacterium and Bacillus [6]. Most of the isolates showed degradation to more than one compound. All the bacteria could tolerate and grow with the compounds at 5–20% (v/v).

Strain Bb5 which belong to Pseudomonas sp showed maximum growth in presence of BTEX compounds Fig 2. Kim et al. [13] have reported that Pseudoxanthomonas spadix isolated from gasoline contaminated soil has the ability to degrade BTEX.

Interestingly, cell viability examination is crucial and necessary to detect bacterial tolerance

because pseudogrowth occurred in treatments of high solvent concentrations. The turbidity of the culture was not the direct result of raised cell concentration and solvent itself show some turbidity after mixing with the media used for the growth

The GC analysis shows that the selected strain Bb5 was able to degrade 100% of benzene [5% (v/v)], ethyl benzene [2% (v/v)], while 80% toluene [5% (v/v)] and 70% xylene [0.5% (v/v)] in 48 h. Alvarez and Vogel [16] reported that together the strains of Pseudomonas sp. CFS-215 and Arthobacter sp. HCB can degrade 50mg/l BTEX concentration to 0.1 mg/l BTEX in 6 weeks. Goudar and Strevett [17] showed that benzene and o-xylene exhibited higher resistance to biodegradation while toluene and p-xylene were rapidly degraded. Ethylbenzene and m-xylene were degraded at intermediate rates. Different concentrations of BTEX, including benzene, toluene, ethylbenzene, and xylene isomers, were biodegraded to non-detectable levels within 70 d by Duo et al. [18] when the initial concentrations were below 100 mg/kg in soil. Increasing the biodegradation capacity of microbial strains would either reduce the volatilization of BTEX to the atmosphere or reduce the need to treat this polluted air stream.

5. Conclusion

The degradation of petroleum hydrocarbons as BTEX compounds is a difficult phenomenon, due to which the disposal and presence of these compounds in the surroundings poses an environmental hazard. The bacterial strain isolated in the present study can tolerate and grow in presence of high concentration of BTEX compounds by degrading it. This characteristic establishes the potential biotechnological application of the strain solvent waste management, oil spills, effluent treatment etc.

References

1. Nicholson A. and B.Z. Fathepure, 2004. Biodegradation of Benzene by Halophilic and Halotolerant Bacteria under Aerobic Conditions. Appl. Environ. Microbiol., 70 (2): 1222-1225.
2. Saeed, T., and M. A. Mutairi, 1999. Chemical composition of the water soluble fraction of the leaded gasolines in seawater. Environ. Int., 25:117–129.
3. Wang, L., and Z. Shao, 2006. Isolation and characterization of 4 benzene / toluene-degrading bacterial strains and detection of related degradation genes. Chin. J. Microbiol., 46(5):753–757 (in Chinese)
4. Arenghi, F.L., D. Berlanda, E. Galli, G. Sello, and P. Barbieri, 2001. Organization and regulation of meta cleavage pathway genes for toluene and o-xylene derivative degradation in Pseudomonas stutzeri OX1. Appl. Environ. Microbiol., 67(7): 3304–3308.
5. Jahn, M. K., S. B. Haderlein, and R. U. Meckenstock. 2005. Anaerobic degradation of benzene, toluene, ethylbenzene, and o-xylene in sediment free iron-reducing enrichment cultures. Appl. Environ. Microbiol., 71:3355–3358.
6. Wang, L., N. Qiao, F. Sun, and Z. Shao, 2008. Isolation, gene detection and solvent tolerance of benzene, toluene and xylene degrading bacteria from near shore surface water and Pacific Ocean sediment. Extremophiles, 12 (3): 1433-4909.
7. Kato, C., A. Inoue, and K. Horikoshi, 1996. Isolating and characterizing deep sea marine microorganisms. Trends Biotechnol 14(1): 6–12.
8. Kim, D., Y.S. Kim, S.K. Kim, S.W. Kim, G.J. Zylstra, Y.M. Kim, and E. Kim, 2002. Monocyclic aromatic hydrocarbon degradation by Rhodococcus sp. strain DK17. Appl Environ Microbiol., 68(7): 3270–3278.
9. Taki H., K. Syutsubo, R.G. Mattison, S. Harayama, 2007. Identification and characterization of o-xylene-degrading Rhodococcus spp. which were dominant species in the remediation of o-xylenecontaminated soils. Biodegradation, 18(1):17–26.
10. Prenafeta-Boldu, F.X., H. Ballerstedt, J. Gerritse, and J.T.C. Grotenhuis, 2004. Bioremediation of BTEX hydrocarbons: Effect of soil inoculation with the toluene-growing fungus Cladophialophora sp. strain T1. Biodegradation, 15: 59–6.
11. Hendrickx, B., W. Dejonghe, F. Faber, W. Boenne, L. Bastiaens, W. Verstraete, E.M. Top, D. Springael, 2006a. PCR-DGGE method to assess the diversity of BTEX mono-oxygenase genes at contaminated sites. FEMS Microbiol. Ecol., 55(2): 262–273.
12. Hendrickx, B., H. Junca, J. Vosahlova, A. Lindner, I. Ruegg, M. Bucheli- Witschel, F. Faber, T. Egli, M. Mau, M. Schlomann, M. Brennerova, V., Brenner, D.H. Pieper, E.M. Top, W. Dejonghe, L. Bastiaens, D. Springael, 2006b. Alternative primer sets for PCR detection of genotypes involved in bacterial aerobic BTEX degradation: distribution of the genes in BTEX degrading isolates and in subsurface soils of a BTEX contaminated industrial site. J. Microbiol. Met., 64: 250–265.

13. Kim, J.M., Le.N. Thuan, B.S. Chung, J.H. Park,., J.W. Bae, E.L. Madsen, and G.O. Jeon, 2008. Influence of Soil Components on the Biodegradation of Benzene,Toluene, Ethylbenzene, and o-, m-, and p-Xylenes by the Newly Isolated Bacterium Pseudoxanthomonas spadix BD-a59. Appl. Environ. Microbiol., 71: 7313-7320.
14. Berlendis, S., J.F. Lascourreges, B. Schraauwers, P. Sivadon, and M. Magot, 2010. Anaerobic Biodegradation of BTEX by Original Bacterial Communities from an Underground Gas Storage Aquifer. Environ. Sci. Technol., 44 (9): 3621–3628.
15. Fong, K.P.Y. and H.M. Tan, 2000. Isolation of a microbial consortium from activated sludge for the biological treatment of food waste. World J. Microbiol. Biotechnol., 16 (5): 441-446.
16. Alvarez, P.J.J., and T.M. Vogel, 1995. Degradation of BTEX and their aerobic metabolites by indigenous microorganisms under nitrate reducing conditions. Wat. Sci. Tech., 31 (1): 15–28.
17. Goudar, C.T. and K.A. Strevett, 1998. Comparison of relative rates of BTEX biodegradation using respirometry. J. Indust. Microbiol. Biotechnol., 21: 11–18.
18. Dou, J., X. Liu, and Z. Hu, 2008. Anaerobic BTEX degradation in soil bioaugmented with mixed consortia under nitrate reducing conditions. J. Environ. Sc., 20 (5): 585-592.

Please Cite This Article As:

Rajni Singh and S. Mary Celin. 2010. Biodegradation of BTEX (Benzene, Toluene, Ethyl Benzene and Xylene) Compounds by Bacterial Strain under Aerobic Conditions. Journal of Ecobiotechnology 2/4 27-32.